In silico analysis of the interactions of ginger actives with the serotonin (5-HT3)receptor

Lohning, Anna Elizabeth; Marx, Wolfgang; Isenring, Elisabeth

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MM2017 - Association of Molecular Modellers of Australasia (AMMA)

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Presenter: Asst/Prof. Anna Lohning
Faculty of Health Sciences & Medicine
Bond University
Gold Coast, Australia

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Clinical study :- Ginger as an effective anti-emetic agent for use in chemotherapy

Marshall, S., McCarthy, A., McKavanagh, D., Vitetta, L., Sali, A.,., Lohning, A., Marx, W., Crichton, M., Reid, K., Isenring, E.

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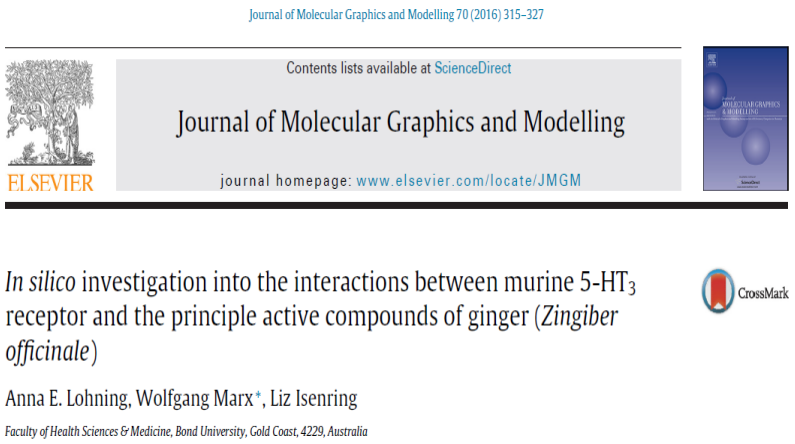
Presentation Overview

- Rationale
- Background / Aim
- Methods
- Results
- Conclusions /
- Planned work



Journal of Molecular Graphics and Modelling 70 (2016) 315–327

Contents lists available at ScienceDirect

Journal of Molecular Graphics and Modelling

journal homepage: www.elsevier.com/locate/JMGM

In silico investigation into the interactions between murine 5-HT_3 receptor and the principle active compounds of ginger (*Zingiber officinale*)

Anna E. Lohning, Wolfgang Marx*, Liz Isenring

Faculty of Health Sciences & Medicine, Bond University, Gold Coast, 4229, Australia

nutrients

MDPI

Article

The Effect of a Standardized Ginger Extract on Chemotherapy-Induced Nausea-Related Quality of Life in Patients Undergoing Moderately or Highly Emetogenic Chemotherapy: A Double Blind, Randomized, Placebo Controlled Trial

Wolfgang Marx [1,2,3,4,*], Alexandra L. McCarthy [5,6], Karin Ried [3], Dan McKavanagh [6,7], Luis Vitetta [8,9], Avni Sali [3], Anna Lohning [1] and Elisabeth Isenring [1,2]

1 Faculty of Health Sciences and Medicine, Bond University, Gold Coast, QLD 4226, Australia; alohning@bond.edu.au (A.L.); lisenrin@bond.edu.au (E.I.)
2 Department of Nutrition and Dietetics, Princess Alexandra Hospital, Brisbane, QLD 4102, Australia
3 National Institute of Integrative Medicine, Melbourne, VIC 3122, Australia; karinried@niim.com.au (K.R.); asali@niim.com.au (A.S.)
4 School of Allied Health, La Trobe University, Melbourne, VIC 3086, Australia
5 Division of Cancer Services, Princess Alexandra Hospital, and Institute of Health and Biomedical Innovation, Brisbane, QLD 4102, Australia; alexandra.mccarthy@auckland.ac.nz
6 School of Nursing, University of Auckland, Auckland 1010, New Zealand; Daniel.Mckavanagh@health.qld.gov.au
7 School of Pharmacy, The University of Queensland, Brisbane, QLD 4072, Australia
8 Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia; luis.vitetta@sydney.edu.au
9 Medlab Clinical Ltd., Alexandria, Sydney, NSW 2015, Australia
* Correspondence: w.marx@latrobe.edu.au; Tel.: +61-03-9479-3069

Received: 30 June 2017; Accepted: 8 August 2017; Published: 12 August 2017

Rationale

- Chemotherapy-induced nausea and vomiting (*CINV*) poses a *major obstacle* to patients. *Variable responses* to current treatments for *CINV* *reduces their effectiveness* providing impetus to *develop more effective treatments* (Hsieh, 2015).

- Clinical trials have shown preliminary support for the *use of ginger* in multiple types of nausea (motion, morning sickness, *chemotherapy-induced*) (Marx, 2013).

- A *key finding* from a double-blinded, randomized-controlled trial (Marx, 2017) in chemotherapy-naïve patients was that intervention participants *reported significantly better* *CINV*-related *quality of life* (*QoL*) & *less fatigue* than placebo participants (Marx et al 2017).

Hsieh, R.K., et al, Support. Care Cancer 2015, 23, 263-272
Marx, W., et al, Nutr. Rev. 2013, 71, 245-254
Marx, W., et al, Nutrients 2017, 9, 867 (Accepted August 2017)

Rationale (cont'd)

- In conjunction with the ongoing clinical studies, we're interested in the *mechanistic aspects* of how *ginger may function* as an anti-emetic.

- *In vitro* studies have shown the active compounds in ginger
  a) Inhibit *serotonin (5-HT_3)-induced contractions* in guinea pig ileum[1]
  b) Inhibit *serotonin-mediated signalling* (possibly in a non-competitive manner)[2]

- Current anti-emetic treatment for ***CINV*** (eg granisetron) *target 5-HT_3 receptors*

- Understanding the details of how ginger actives bind and interact with this receptor will help guide the design for *more effective treatments*.

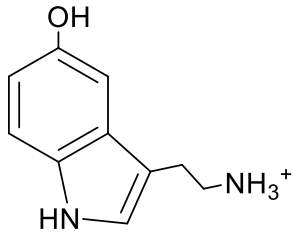
1 Pertz, J. et al Planta Med. 77 (10) (2011) 973–978
2 Walstab, J.et al Neurogastroentereol. Motil., 25 (2013) 439-447 (e302);
2 Abdel-Aziz,H. et al Planta Med. 71 (2005) 609–616.

Introduction

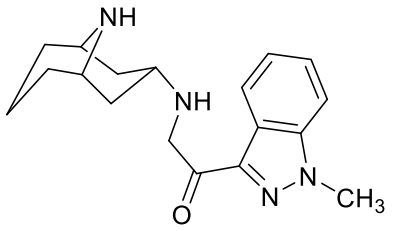
- A primary pathway for emesis relating to ***CINV*** is stimulation of the vagal afferent nerves causing release of high levels of serotonin (5-HT_3)

- Serotonin binds to receptors on afferent nerves sending a signal to the central nervous system, mediating a range of physiological functions.

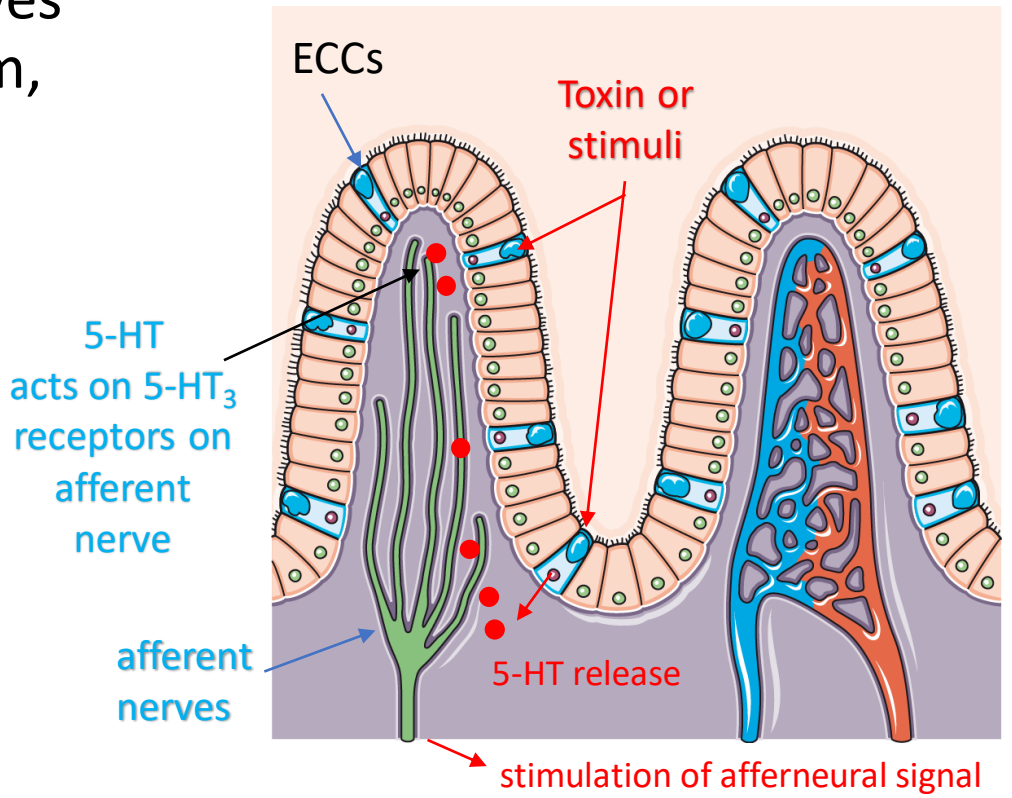
- Current treatment for ***CINV*** involves use of anti-emetics (***setrons***) that *competitively inhibit* 5-HT_3 receptors thus decreasing 5-HT response.



Serotonin



granisetron

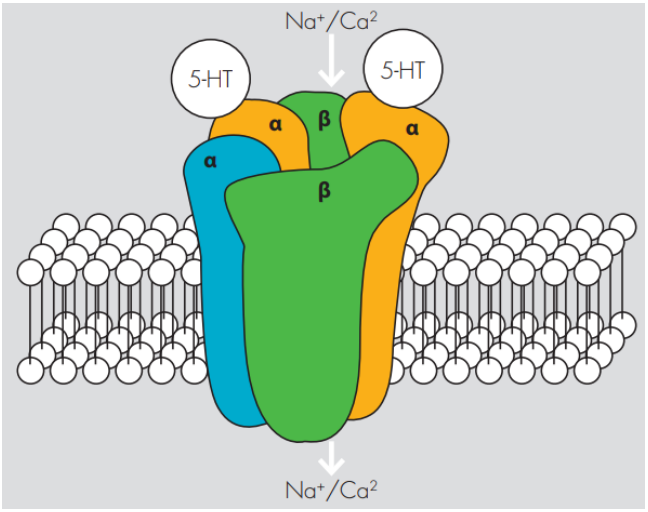


Stimuli includes luminal distension, parasympathetic innervation or changes in osmotic concentrations in intestinal contents.

ECCs = enterochromaffin cells

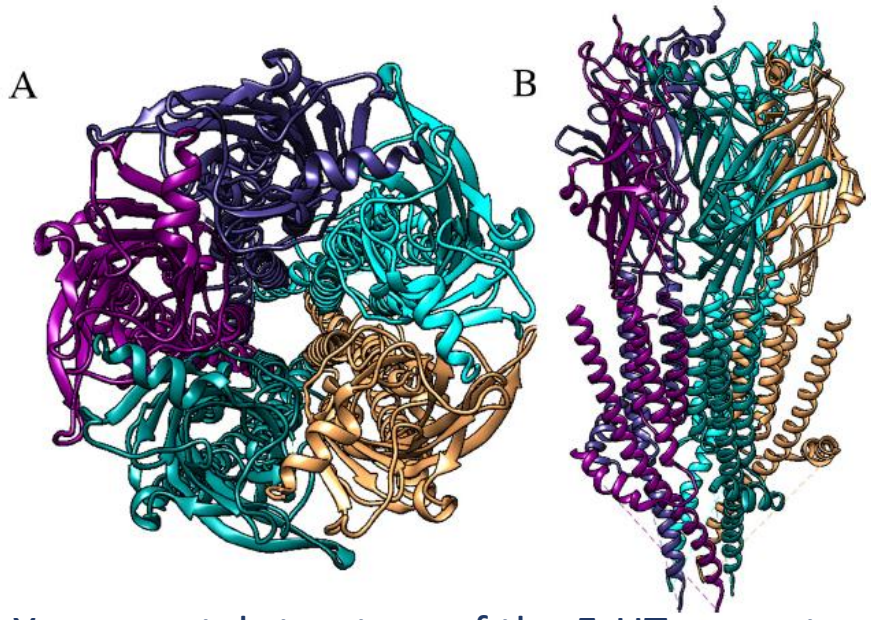
Introduction (cont'd)

- The 5-HT_3 subtype of serotonin receptors are cationic, *pentameric ion channels*. Other examples of this receptor type include GABA, glycine, nAch receptors.



- 5 distinct subunits (5-$HT3_{A \rightarrow E}$) leads to *complexity of function*. (eg Zn^{2+} & small alcohols effect functional state of receptor.

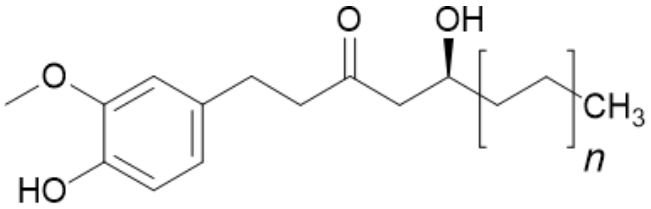
- Functionally, the channel can be either open, closed or desensitized – serotonin binds with high affinity to the *open* channel but



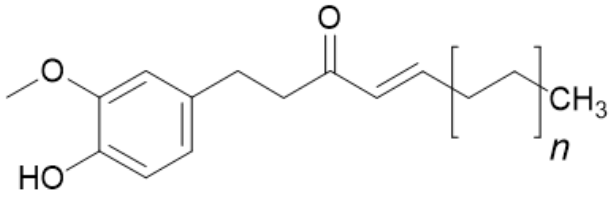
X-ray crystal structure of the 5-HT_3 receptor (4pir.pdb) (Hassaine 2014) A (top); B (side)

Introduction (cont'd)

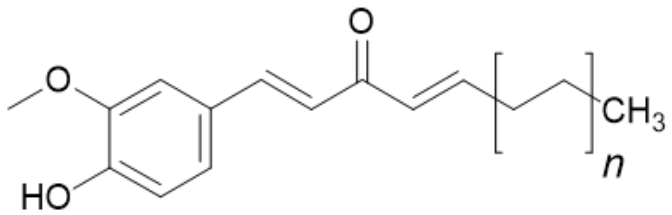
- *Gingerols* are the primary bioactives within the non-volatile, pungent component of the ginger rhizomes (*Zingiber officinale*).



Gingerols (n=6,8,10)



Shogaols (n=6,8,10)



Dehydoshogaols (n=6,8,10)

- *In vitro* studies by Abdel Aziz in 2005 found that 6S, 6G, 8G and 10G *inhibited 5-HT_3-induced contractions* of the isolated guinea-pig ileum.
- Since they were unable to displace ^{3}HGR65630 (a competitive inhibitor) a *non-competitive mechanism was proposed (potential allosteric site)* Similar findings were reported by Walstab in 2013.
- However the mechanism remains unclear[1,2]

1 Ryan, J.L., et al Support. Care Cancer 2- (2012) 1479-1489.
2 Marx , W. et al Curr. Opin. Support, Paliat. Care 9(2) (2015) 189-195.

Aims

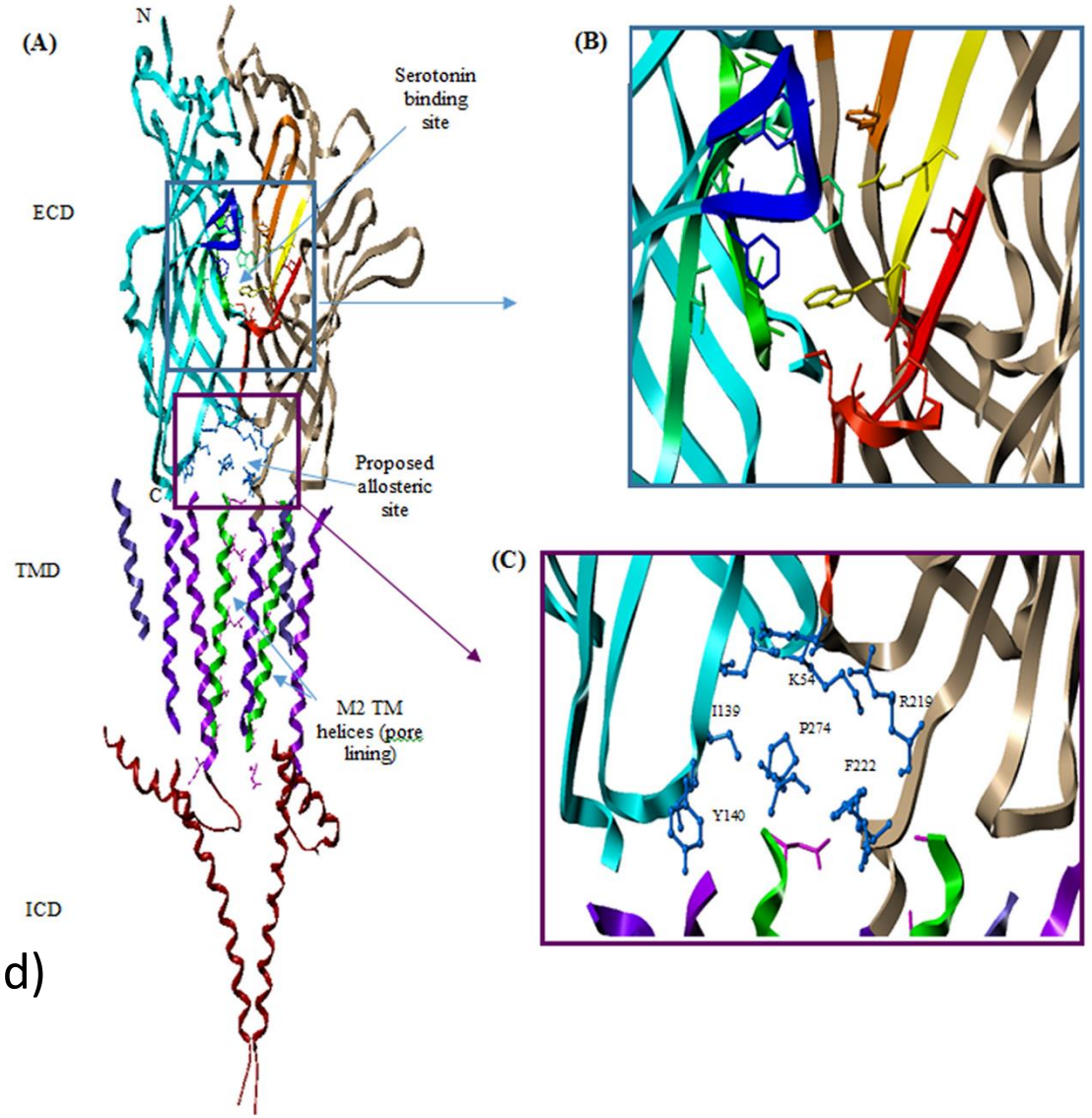
1. Given there is no ligand-bound crystal structure to date, we aim to probe the *serotonin* and *proposed allosteric sites* with a range of *in silico* techniques that may suggest ginger actives may play a role at the 5-HT_3 receptor

2. To compare the stability of 6-gingerol, serotonin and granisetron in each site using molecular dynamics simulations.

Method (Part 1):

Target preparation

- Homopentameric mouse 5-HT_3 receptor (4pir.pdb)
- Both serotonin & allosteric sites are located at interface of two subunits (principle/complementary) with key interacting residues from both subunits (A_PA_C) extracted
- 2 subunits (A_PA_C) extracted for analysis (ECM/TM/ICD) *
- Energy minimized (Gast-Hückel charges & H added)



* SYBYLx2.1.1 molecular modelling software

Method (Part 1):

Ligand database preparation

- Structures obtained either from Pubchem/PDB databases or prepared in ChemDraw.

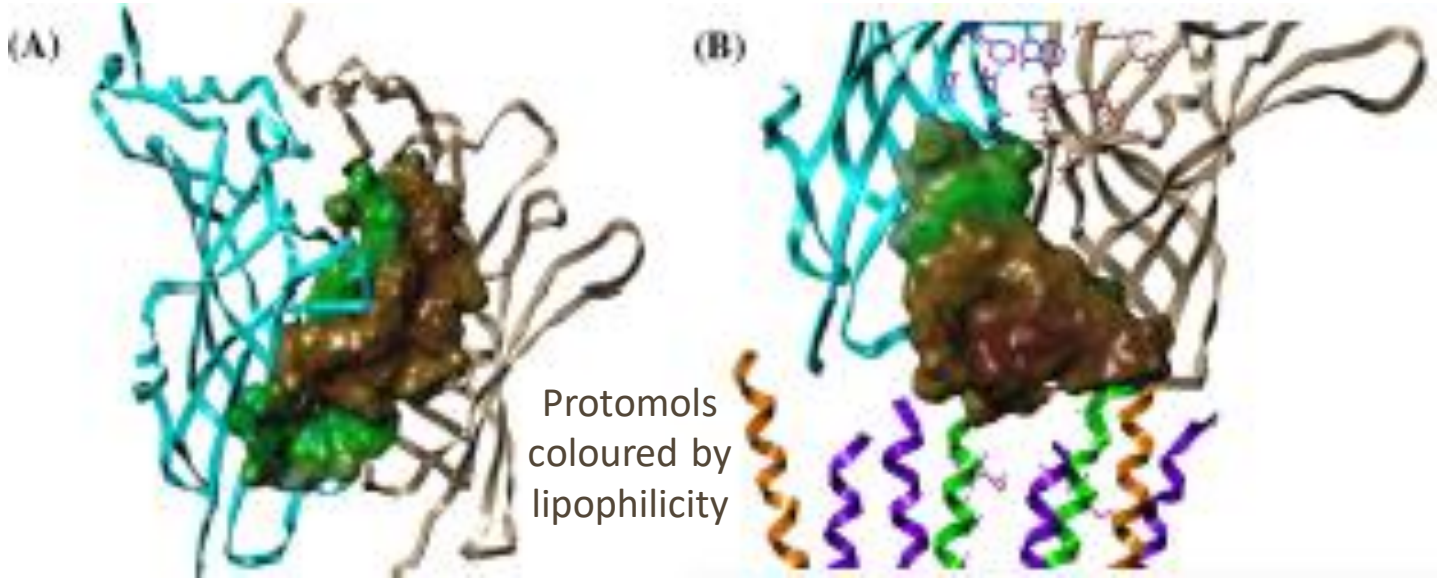
Ligand	Type
Serotonin, (5-HT)	cognate ligand
6,8,10-G 6,8,10-S 6,8,10-DHSG	Gingerols Shogaols Dehydroshogaols
capsaicin, curcumin	Structural analogs of ginger actives
granisetron, ondansetron, etc	Positive Controls (5-HT site) (Setrons) (competitive)
PU02, bicurculline, etc	Positive Controls (allosteric site) (non-competitive)
Acetylcholine, GABA	Negative Controls (Decoys)

* Energy minimization Protocol	
Forcefield	Amber FF99 Amber atom types
Charges	Gasteiger-Huckel
Method	Steepest Descent
Convergence	0.5 kcal/mol

Method (Part 1):

Molecular Docking (Surflex-Dock 2.1)

- Protocol: Serotonin site (multi-channel) Allosteric site (residue-based)
- “Flexible” docking approach (ligand & protein atoms around site of interest).
- Poses ranked according to Total Score ($1/K_d$) loosely approximating a ***theoretical*** binding affinity.
- *C-score* validation. Compares 4 scoring functions each with different weightings for non-bonded interactions)

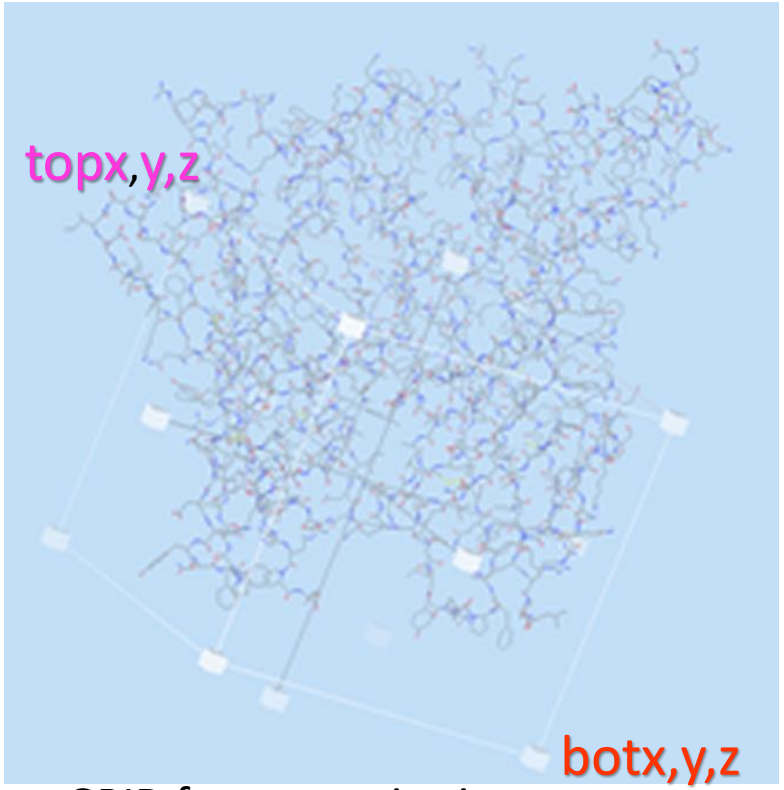


serotonin site (A) allosteric site (B)

Method (Part 1):

2. GRID Analysis

- Interaction energies calculated at each grid point (kcal/mol) (Goodford, 1985).
- Grid box (dimensions (topx,y,z; botx,y,z)) generated around each site. (0.33 Å resolution)
- A set of small atomic/molecular probes was selected to mimic the chemical properties of key functional groups of the ligands.



GRID for serotonin site

	Serotonin Site	Allosteric site
Bottom	144.82	138.06
Top	181.15	184.06
Y	157.57	166.93
y	193.9	209.93
Z	231.82	250.75
z	277.82	293.75

3. Sequence Alignment

- ClustalOmega alignment between mouse and human 5-HT_3 receptor sequences was performed to identify the degree of homology and identify conservation of residues likely to be important in ligand binding.

Goodford, P. J. Med.Chem. 28 (7) (1985) 849–857.

Method (Part 2):

Molecular Dynamics Simulations

- Target Preparation
  - Initially a solvated dimer (A_PA_C), ECD domain in dodecahedron box (SPC water)
  - Gromacs-5.04 (FF – gromos54a7_FF – gromos54a7)
- Ligand Preparation
  - Topologies obtained from ATB[1] & superimposed onto docked ligand pose.
- Usual preparation prior to full MD production run
  - EM (steepest descent, 1000 steps, conv.
  - NVT ensemble - Canonical isothermal thermostat (Berendson temp coupling)
  - NPT ensemble - barostat
  - MD – 10ns, 2fs ts

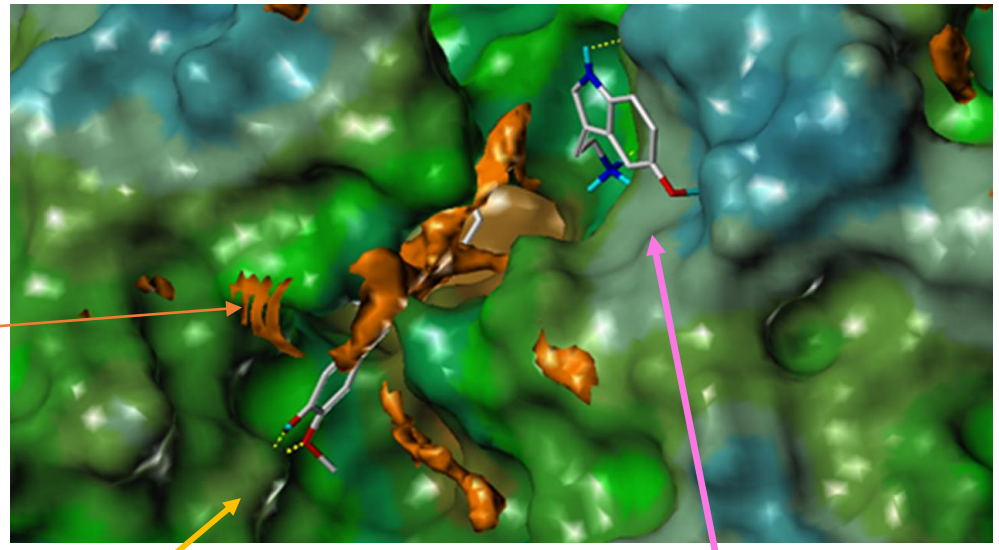
Key MDP Parameters
Neighbour coupling (Verlet) E'statics
(Reaction-field, epsilon = 78)

[1] *Malde AK, Zuo L, Breeze M, Stroet M, Poger D, Nair PC, Oostenbrink C, Mark AE.* An Automated force field Topology Builder (ATB) and repository: version 1.0. *Journal of Chemical Theory and Computation*, 2011, 7(12), 4026-4037

Results – Molecular Docking

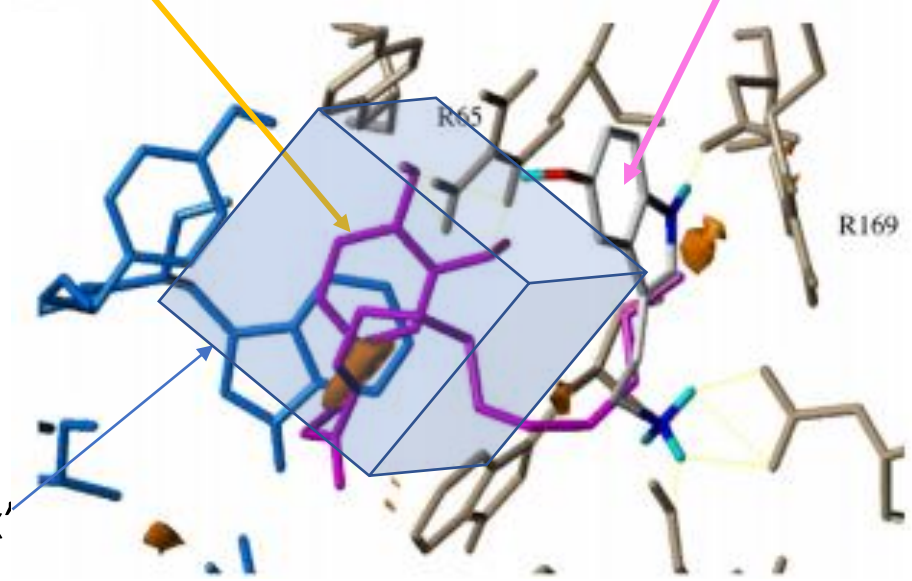
Serotonin site

- GRID analysis & Connolly surface (top) show lipophilicity nature of serotonin site.
  - orange contours (GRID 1.5 kcal/mol, strong interactions with hydrophobic probe)
  - Serotonin (total score 5.7) and 10G (total score 10.81) docked into the serotonin binding site.
- Top scoring 10G (& all other ligands) docked into a location *distinct and more hydrophobic* than that of serotonin.
- Residues previously thought to be important for binding serotonin: S176, R65, D42
- Additional residues found to interact with setrons and ginger compounds: E173, D177 (E209 (granisetron)
  - Position of key residues forming 'aromatic box' (Y207, W156 P subunit; Y127, W63 C subunit)



10-gingerol

serotonin



Results:

Sequence alignment (ClustalOmega)

- subunits A and B of the mouse & human 5-HT_3 receptors

- Key residues highlighted for :-
  - principle subunit (blue shaded box)
  - complementary subunit (grey shaded box)
  - pore-facing residues of TM2 (red star *)
  - TM regions M1-M4 (underlined).

- Results show human & mouse 5-HT_{3A} share ~85% sequence homology while 5-HT_{3B} share ~73%. Human A & B subunits share only ~44%.

Key Finding:
All residues required for stabilising ginger compounds in both sites were conserved between mouse & human

FASTA colouring scheme (based on residue type)

Results – Summary of Molecular Docking

Sorted by clogP **Serotonin site**

compound	clogP	Total Score SERO	Total Score ALLO
10-S	5.9	9.34	8.29
8-DHSG	5.7	8.56	6.61
10-G	5.3	10.8	8.26
6-DHSG	4.6	6.97	6.28
6-S	3.7	8.31	6.52
PU02	3.7	5.8	4.33
capsaicin	3.6	8.54	9.23
curcumin	3.2	8.77	7.02
bicurculline	2.6	7.09	6.01
6-G	2.5	8.7	8.26
VUF1066	2.4	5.13	5.8
ondansetron	2.1	5.22	4.85
granisetron	1.5	5.51	4.87
varenicline	0.8	5.09	4.23
picrotoxin	0.5	4.77	4.96
serotonin	0.2	5.63	6.02
ginkgolide	-0.4	4.25	3.94
GABA	-3.2	4.9	4.76
acetylcholine	-3.7	4.9	4.98

Sorted by Total Score **Serotonin site**

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10-G	5.3	10.8	8.26
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ondansetron	2.1	5.22	4.85
VUF1066	2.4	5.13	5.8
enicline	0.8	5.09	4.23
acetylcholine	-3.7	4.9	4.98
GABA	-3.2	4.9	4.76
picrotoxin	0.5	4.77	4.96
ginkgolide	-0.4	4.25	3.94

Sorted by Total Score **Allosteric site**

compound	clogP	Total Score SERO	Total Score ALLO
capsaicin	3.6	8.54	9.23
10-S	5.9	9.34	8.29
10-G	5.3	10.8	8.26
6-G	2.5	8.7	8.26
curcumin	3.2	8.77	7.02
8-DHSG	5.7	8.56	6.61
6-S	3.7	8.31	6.52
6-DHSG	4.6	6.97	6.28
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PU02	3.7	5.8	4.33
varenicline	0.8	5.09	4.23
ginkgolide	-0.4	4.25	3.94

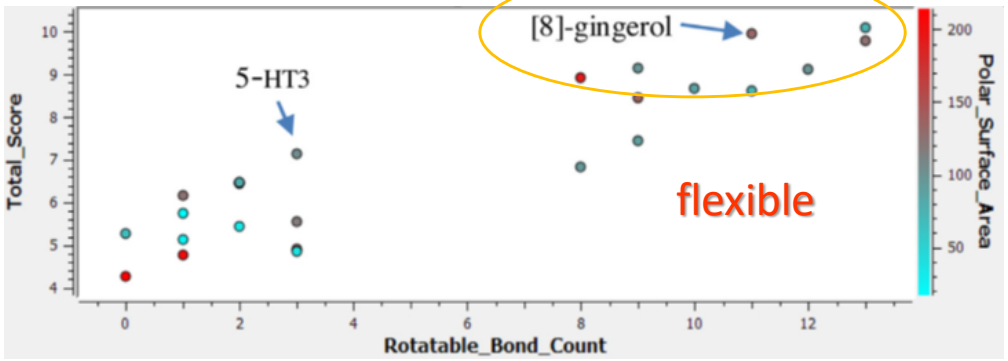
- **Serotonin** scored mid field in both sites (polar)
- Ginger compounds scored high in both sites (as did **structural analogs** (all amongst most hydrophobic)
- **Competitive antagonists** scored mid field at both sites (very similar clogPs)
- **Polar non-competitive antagonists (NCAs)** scored lowest in serotonon site. The more lipophilic NCAs scored higher in serotonin site. (Nb. allosteric modulators are more potent in heteromeric receptors)
- **Decoys** (highly polar) scored poorly in both sites. (Most polar scored mid range in allosteric site)

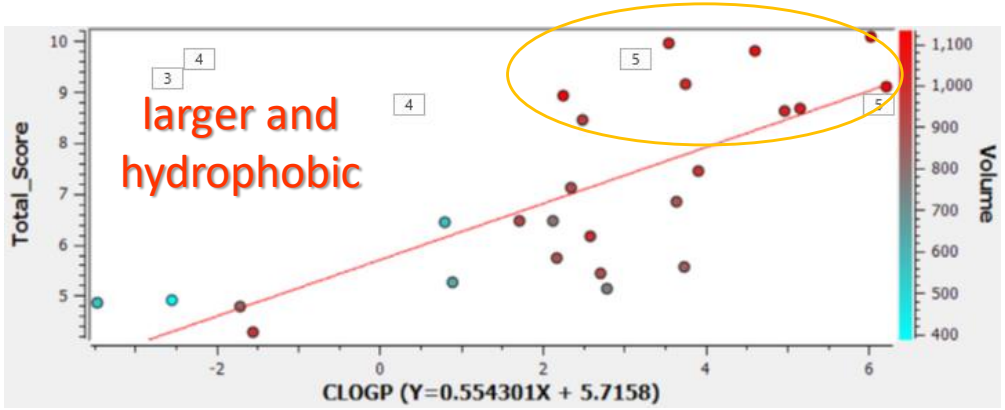
Polarity was a key factor for binding in serotonin site than the allosteric site

Results – Molecular Docking

Serotonin site

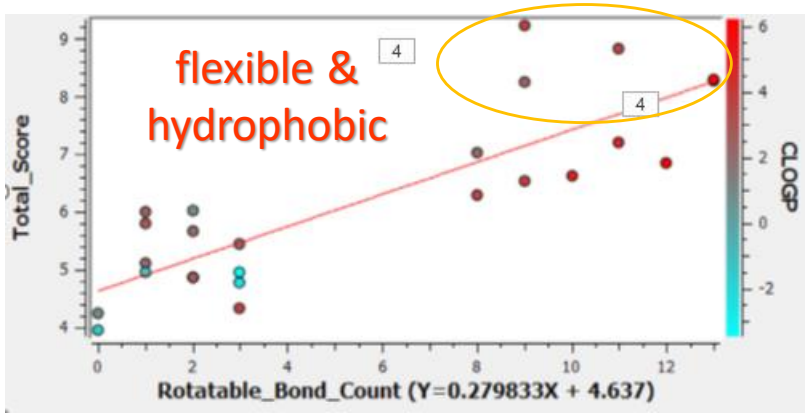
- Ligand flexibility played a more important role than PSA in scoring
- Compounds scored high that were :-

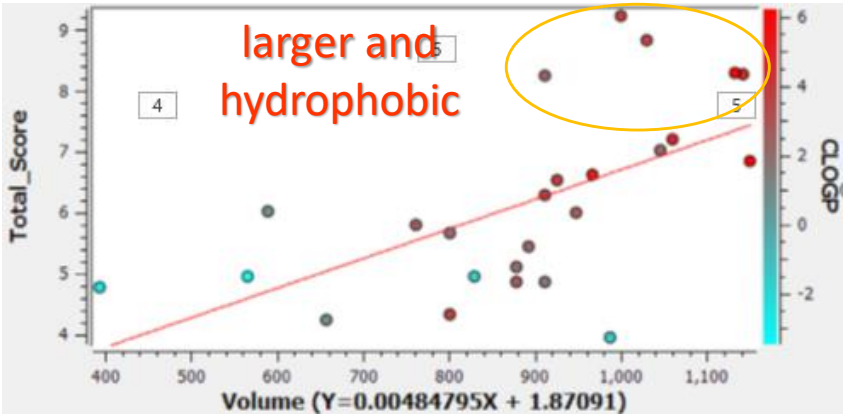




Allosteric site

- Ligand lipophilicity (clogP) & flexibility / size were positively correlated.
- Compounds scored high that were :-





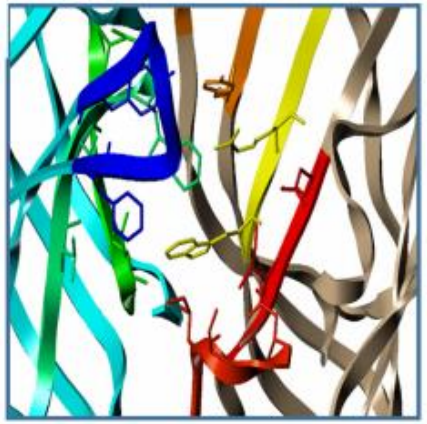
Results – Molecular Docking

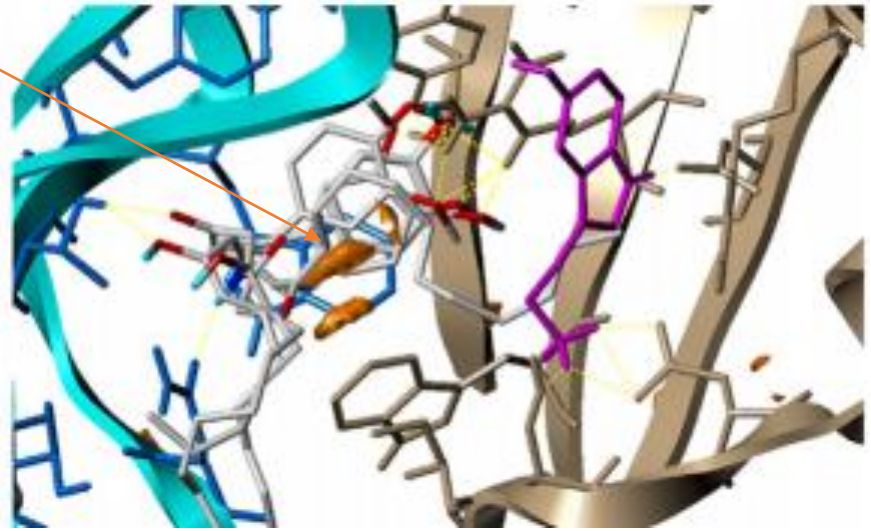
Serotonin site

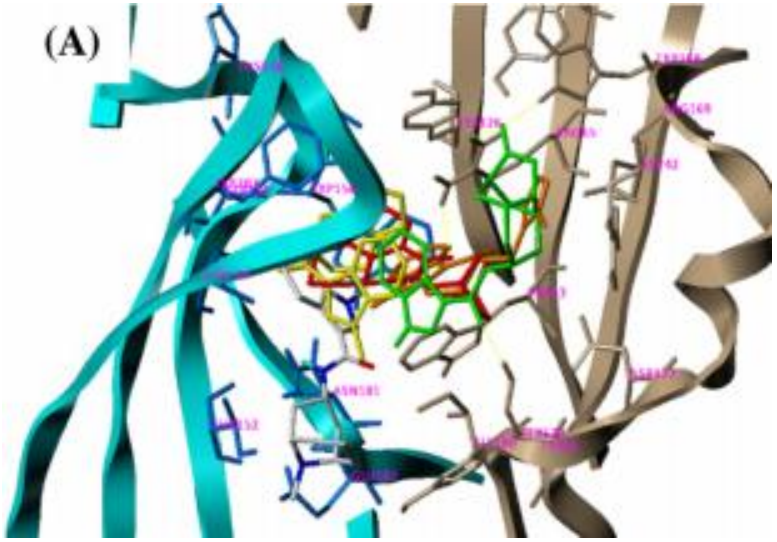
- Our results confirmed the importance of key residues thought to stabilise serotonin in this site, especially R65, N101, T154.
- Our results identified novel interactions with serotonin (D177, E173) and dolasetron (E209) and gingerols (K211, E209, L157)
- GRID successfully predicted position of aromatic ring of docked ginger actives.

PS
Loop A (N97, N101)
Loop B (T53, T54, W156)
Loop C (F199, Y207)

CS
Loop D (W63, R65, Y68)
Loop E (Y124)
Loop F (D177, S178, V180)







Granisetron (atom colours) **ondasetron**; **dolasetron**; **romasetron**; **palonosetron**

Results – Molecular Docking

- Serotonin site – total scores ranged from 4.25-10.81 (-logK_D)
  - 10G scored highest (ginger actives & structural analogs scored highly)
- Allosteric site – total scores ranged from 3.94-9.23 (-logK_D)
  - Capsaicin (structural analog) scored highest followed by gingerol compounds in *allosteric site*
- Experimental IC_{50} data (where available) included for comparison with docking scores for highest binding pose/ligand.

		Serotonin Site				Allosteric Site			
Compound	IC_{50}	Total score (-logK_d)	Cscore	Hbonds[b]	Interacting Residues[c]	Total score (-logK_d)	Cscore	Hbonds[b]	Interacting Residues[c]
Ginger Compounds									
6G	30 *u*M (rat)[i]	8.7	1	3	E209 R65	8.26	1	4	E219 Q56 F222 E53
8G	*u*M range[ii]	10.25	5	4	T154 E209 R65	8.84	5	3	E53 R219 F222
10G	*u*M range[ii]	**10.81**	4	5	T154 E209 K211 T152	8.26	1	5	T280 I139 E53 Q56
6S	9,3 *u*M (rat)[i]	8.31	0	2	N101 W156	6.52	0	3	E53 F222 Q56
8S	*u*M range[ii]	9.06	5	4	R65 S155 T154	7.19	2	2	K54 F222
10S	*u*M range[ii]	9.34	2	2	T152 N101	8.29	5	1	F222
6DHSG	-	6.97	0	3	T152 N101 K211	6.28	0	3	E53 Q56 K54
8DHSG	-	8.56	0	3	L157 N101 Y207	6.61	0	1	E186
10DHSG	-	9.07	2	2	L157 N101	6.85	4	3	E53 Q56 K54
Endogenous Ligand									
serotonin	7.8 uM[a,i]	5.63	4	5	E173 S176 D42 D177	6.02	0	4	Q184 E53 D138 L137
Structural Analogues of ginger actives									
Capsaicin	-	8.54	0	4	R65 N101	**9.23**	1	3	K54 R219 F222
Curcumin	-	8.77	0	9	R65 T154 S155 D177 S179	7.02	0	3	R219 E53 E186

Residues in blue (previously suggested by Hassaine to be important for stabilising serotonin

Results – Molecular Docking

- The setron family of anti-emetics ranked mid-field at both sites
- Non-competitive ligands scored poorly as did decoys. (Nb. Allosteric ligands are observed to be more potent towards heteromeric targets)

- Cscores were high for 10G indicating a *consensus* between scoring functions for their *overall ranking*.

- Cscores were similarly high for serotonin, some setrons & non-competitive ligands.

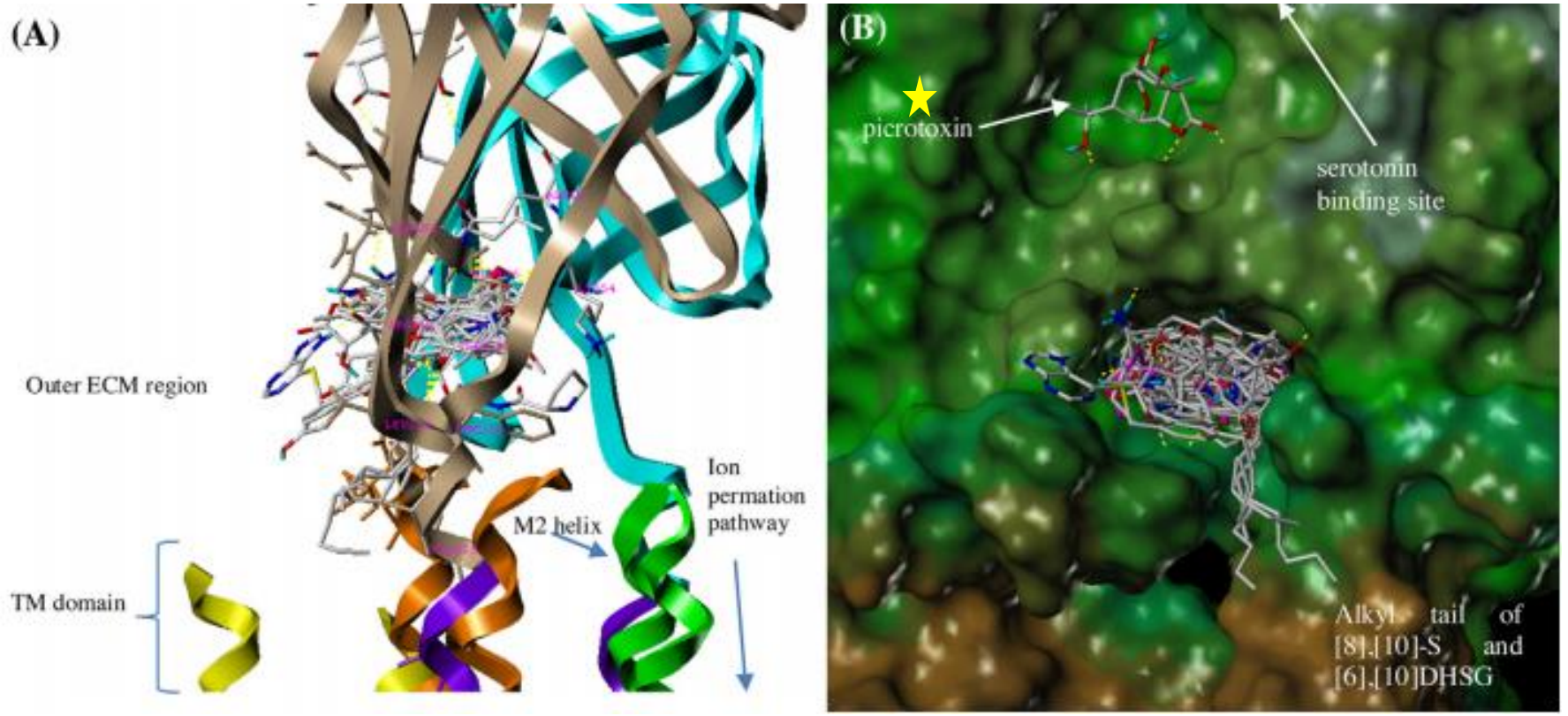
		Serotonin Site				Allosteric Site			
Compound	**IC_{50}**	**Total score (-logK$_d$)**	**Cscore**	**Hbonds[b]**	**Interacting Residues[c]**	**Total score (-logK$_d$)**	**Cscore**	**Hbonds[b]**	**Interacting Residues[c]**
Competitive Antagonists									
Ondansetron	4.9 nM (human)	5.22	5	1	T154	4.85	0	1	Q56
Granisetron	1.4 nM (human)	5.51	5	1	E209	4.87	0	0	-
Palonosetron	31.6 nM (rat)	5.74	0	1	R65	5.1	0	0	-
Dolasetron	20.03 nM (NG108-15)	6.9	0	3	R65 T154	5.43	1	0	-
Ramosetron	11-12 nM (human)	6.48	4	1	T154	5.65	2	2	P274 Q56
VUF10166[41]	40nM (AB subunit only)	5.13	5	1	R65	5.8	4	0	-
Agonist (non-specific)									
Varenicline[43]	5.9 uM[42] (EC_{50})	5.09	4	2	R65 N101	4.23	3	1	P274
Non-Competitive Ligands									
PU02	1.3 *u*M (human)	5.8	5	3	D177 S179	4.33	2	1	D138
Bicuculline	191 *u*M[44]	7.09	5	1	R65	6.01	1	3	-
Picrotoxin	440 *u*M[44]	4.77	5	4	E102 S150 S136 N148	4.96	0	4	Y46 N183 S136
Ginkgolide	727 *u*M[44]	4.25	2	7	K211 S150 E102 T152 N101	3.94	3	3	T280 D138 I139
Decoys									
Acetylcholine	-	4.9	0	0		4.95	3	1	-
GABA	-	4.9	4	3	W156 R65	4.76	1	3	-

Results: Molecular Docking

Allosteric site

Allosteric modulation permits fine-tuning of ion permeation via signal dampening.

The larger volume allows gingerols to adopt a more extended conformation facilitating favourable hydrophobic interactions with the transmembrane region.



★ Picrotoxin (NCA) is able to differentiate between A & B subunits[1].

1 Thompson, A.J. et al Trends Pharmacol. Sci. (2013) 34(2), 100-109

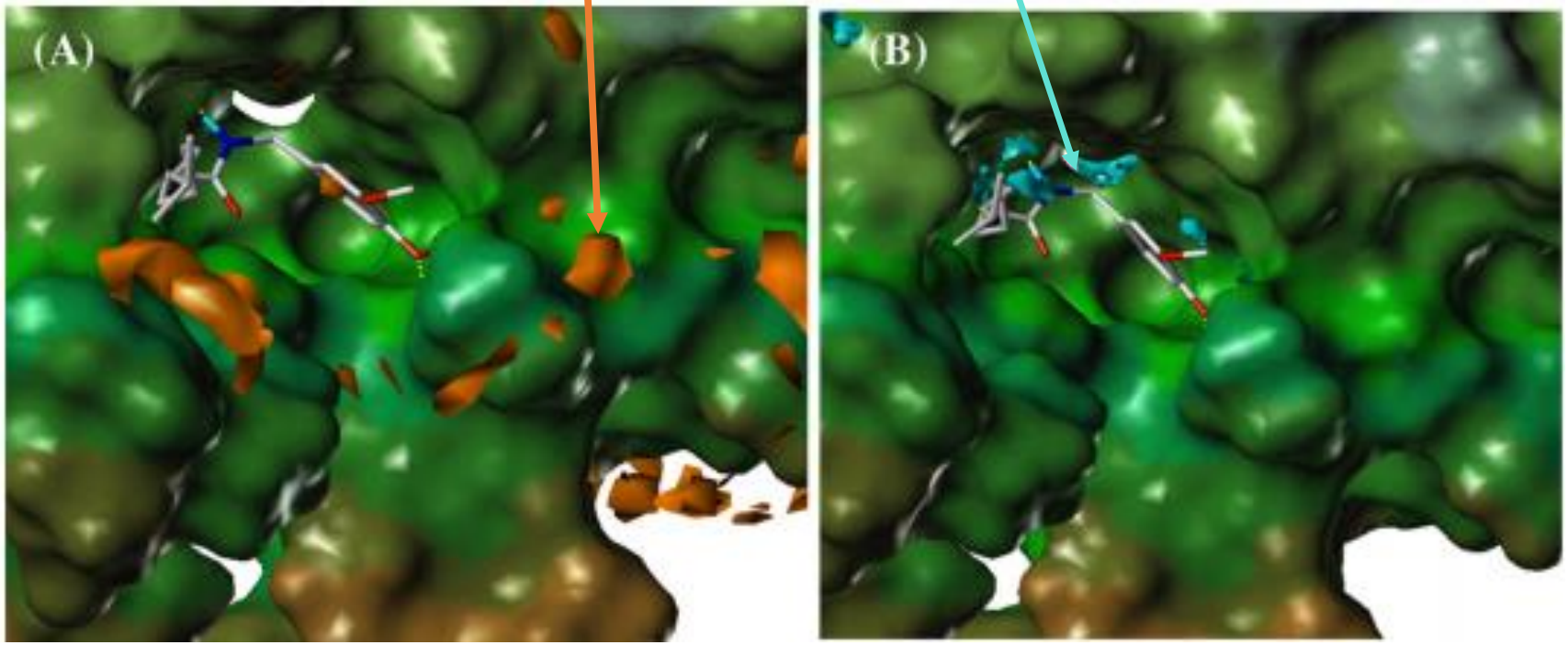
Results: Molecular Docking

Allosteric site

Top scoring ligand, capsaicin. Ginger actives also score well.
This site was found to be more hydrophobic compared to the serotonin site.

(A): GRID contours for a hydrophobic probe (-0.5 kcal/mol).

(B): water probe (–11 kcal/mol) coincides with polar groups



Connolly surface coloured by lipophilic character

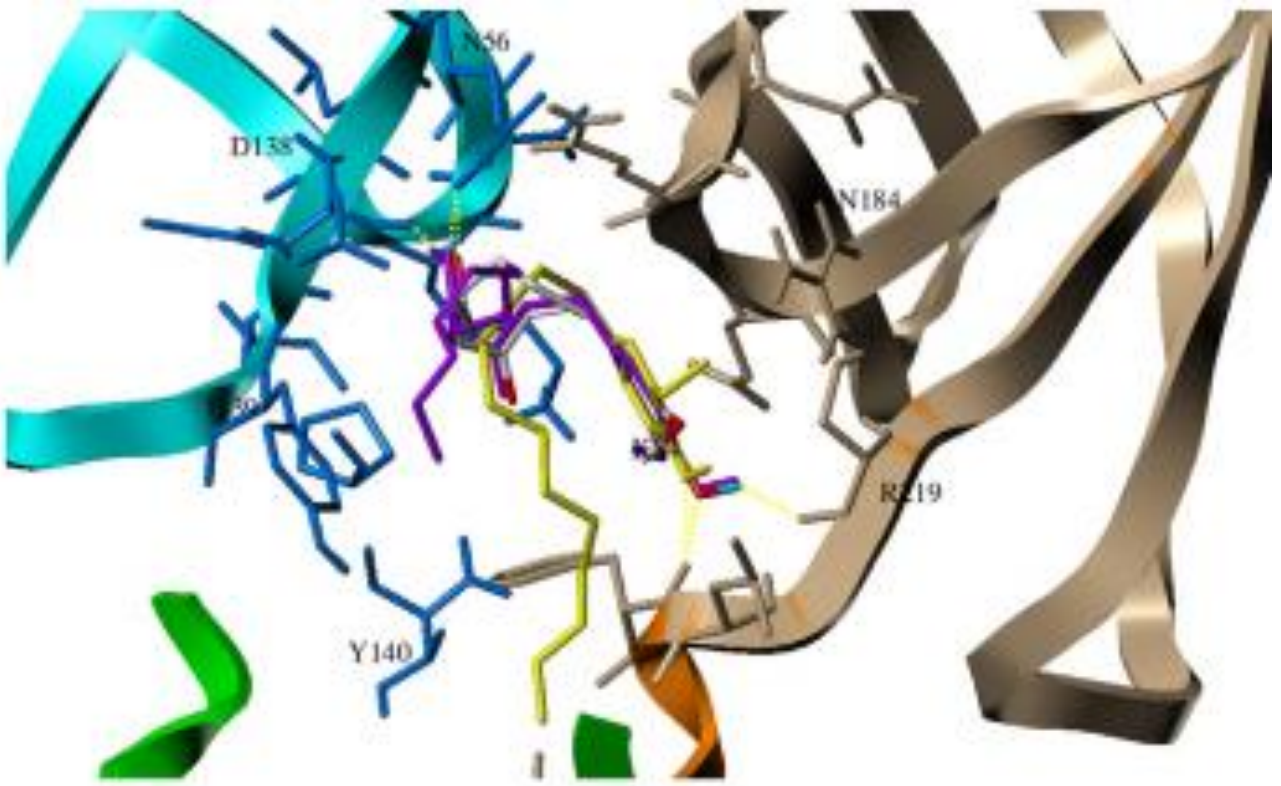
Results: Molecular Docking

Allosteric site

- Ginger actives ranked highly.

  Gingerols > shogaols > DHSGs

- Order correlates with the higher polarity of the site.
- Unlike serotonin site, polarity was not the key determinant contributing to score
  - Eg. PU02 (clogP similar to ginger actives) scored low)



Superimposition of 6G, 8G and 10G

<u>Key Finding</u>:

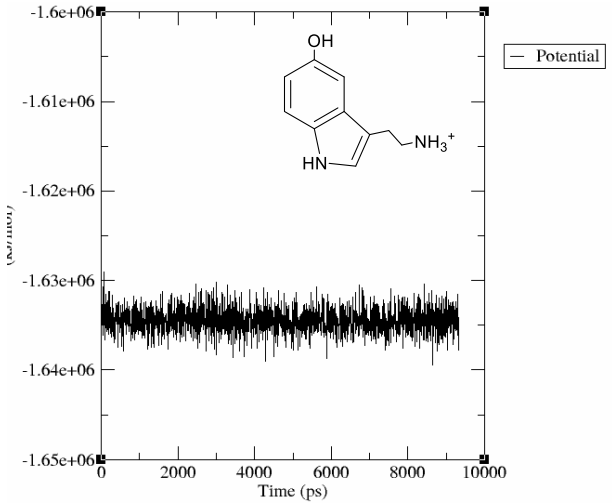
Flexibility and hydrogen bonding capacity played a key role in binding interaction

Potential Energy over 10ns simulation

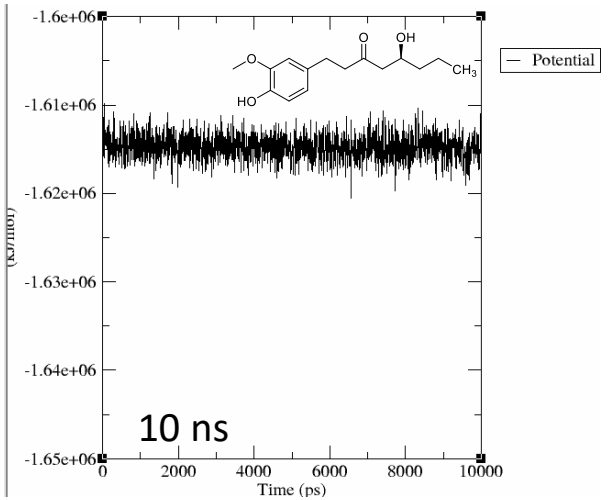
Serotonin Site

Allosteric Site

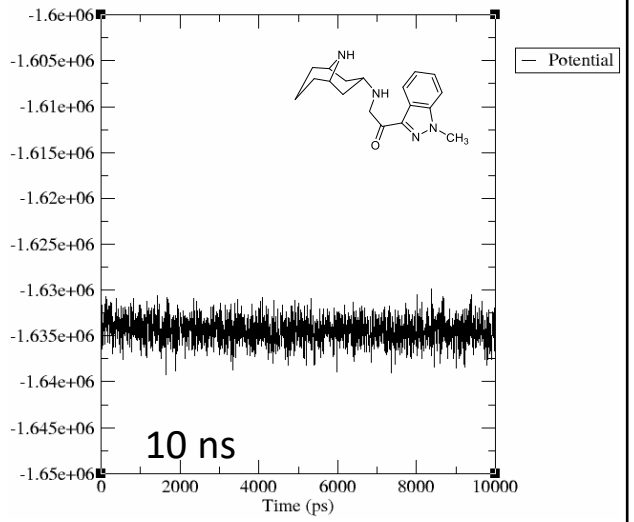
serotonin



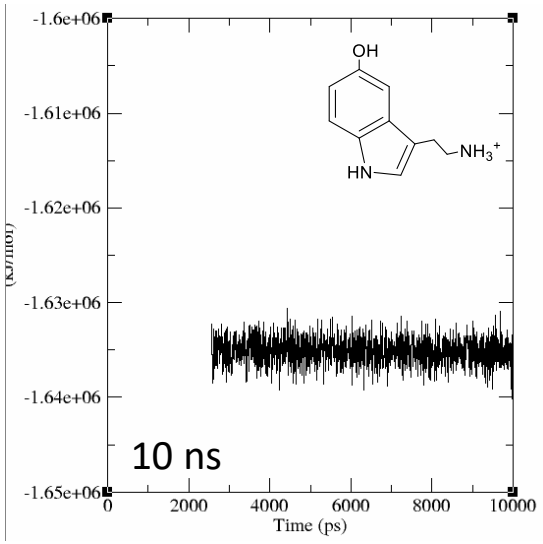
6-gingerol



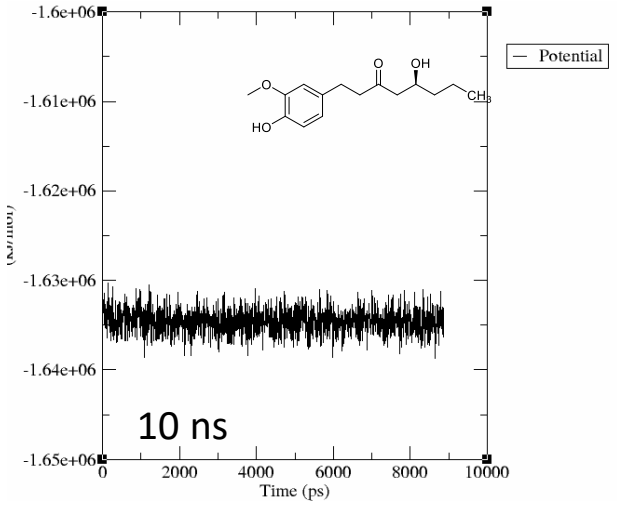
granisetron



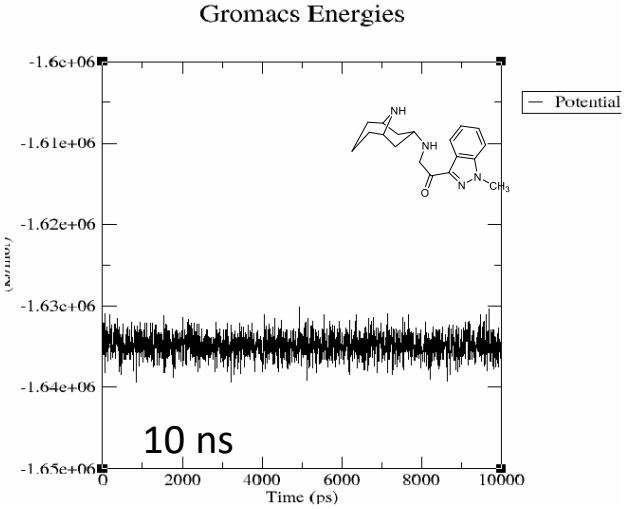
serotonin



6-gingerol



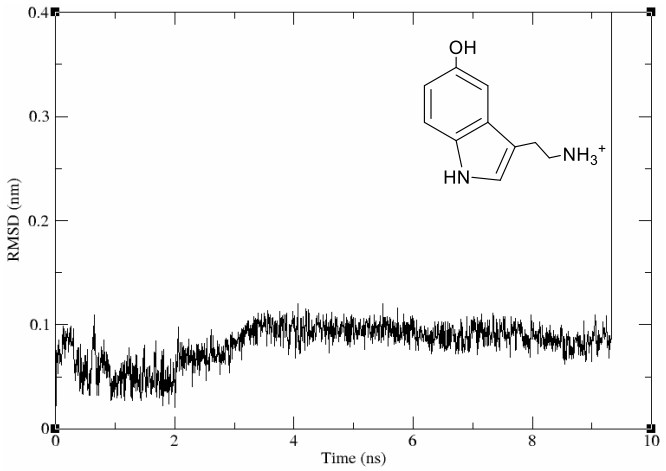
granisetron



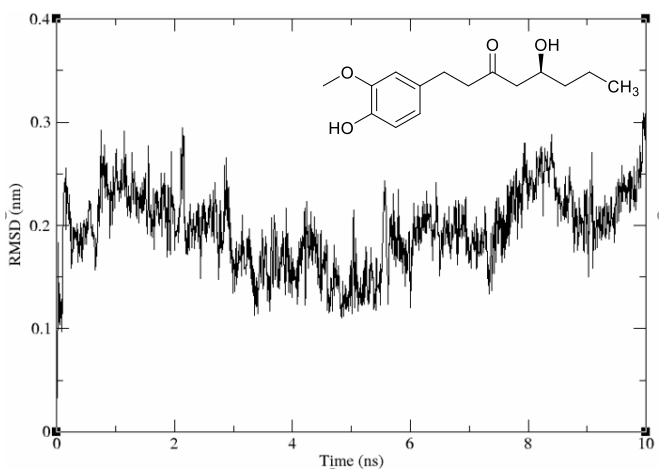
RMSD Comparison of Ligand Stability

Serotonin Site

serotonin



6-gingerol



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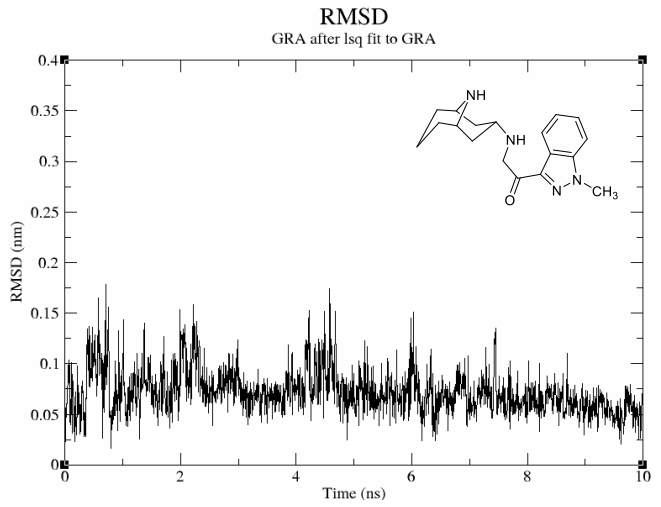
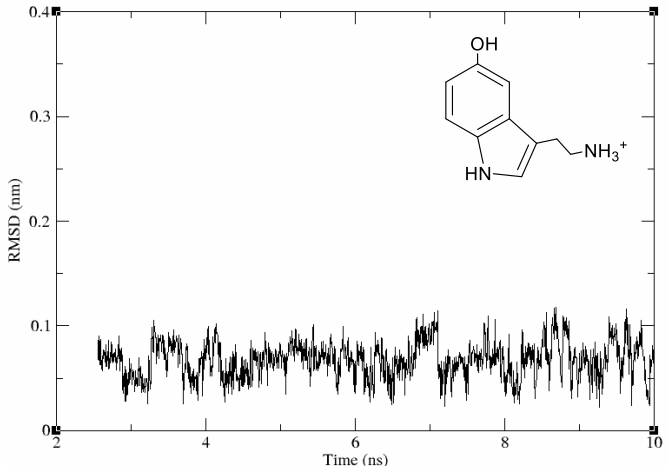
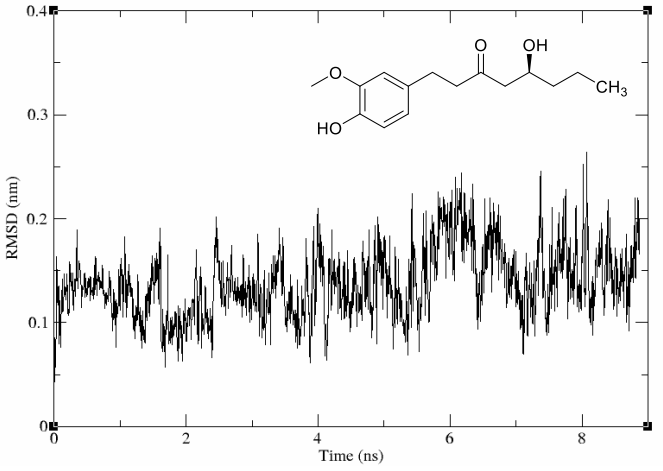


Allosteric Site

serotonin



6-gingerol



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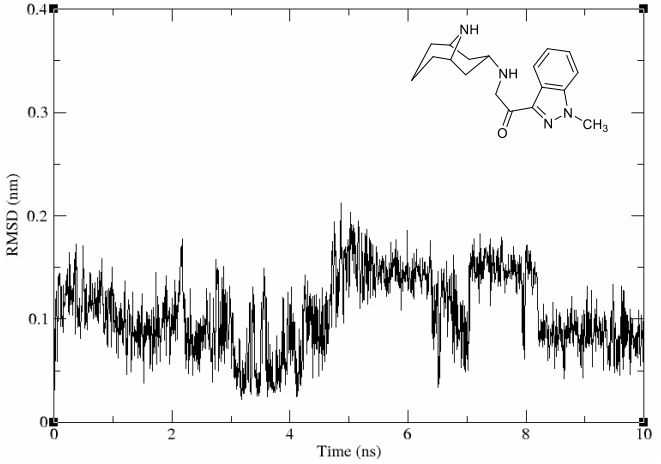
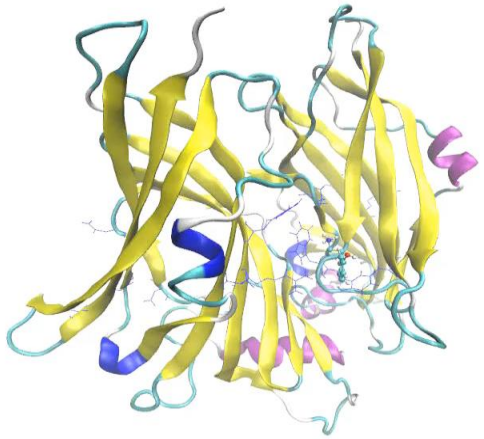
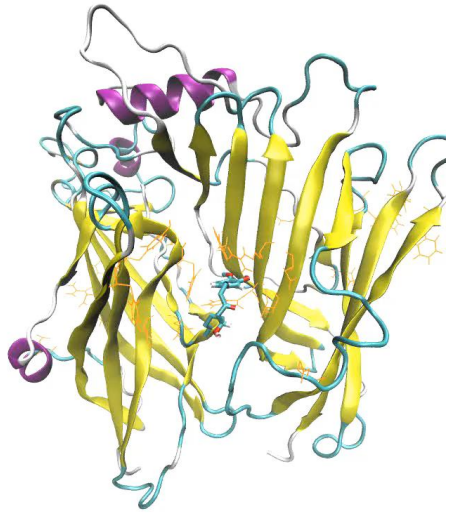
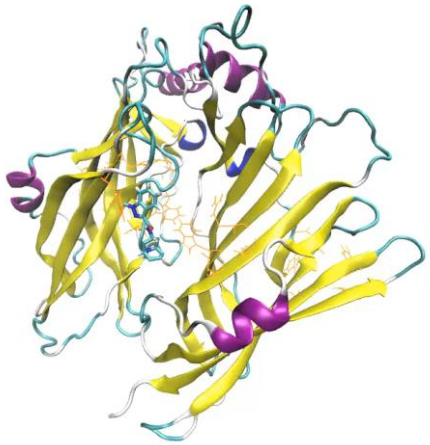
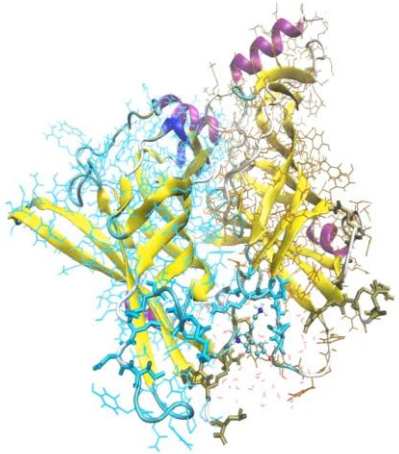
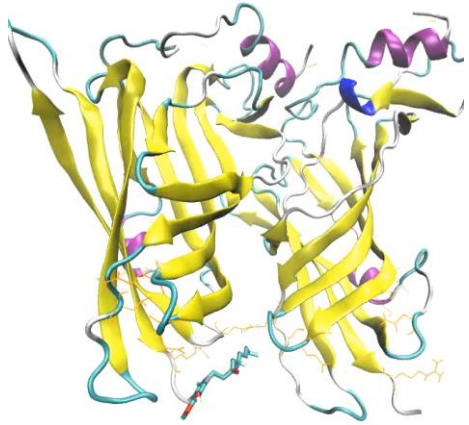
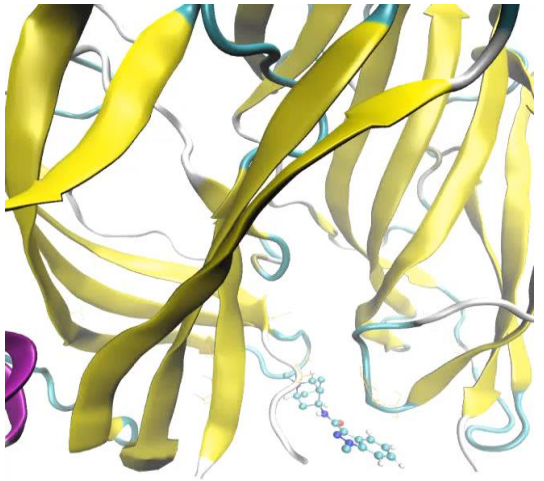


Trajectories

Serotonin Site	serotonin 	6-gingerol 	granisetron 
Allosteric Site	serotonin 	6-gingerol 	granisetron 

Conclusion from MD analysis … *to date*

Limitations

- Species differences
- Functional State
- Subunit Composition
- Transmembrane/ECM interface - another potential binding site
- Inherent in Molecular Docking approaches are
  - Inaccuracies in the energy models used to score potential ligand/receptor complexes
  - The inability of current methods to account for conformational changes that occur during the binding process not only for the ligand, but also for the receptor (ie. how to cope with protein flexibility (1000's of degrees of freedom)
  - The above can be alleviated by using the more robust, Molecular Dynamics (full protein flexibility) – see later.

Conclusions / Future Directions

Key Findings

- Serotonin bound to a site distinct from other ligands in serotonin site. This correlated with site hydrophobicity (.
- Ligand hydrophobicity directly correlated to higher scoring in serotonin site while ligand flexibility and hydrogen bonding capacity facilitated more potent interactions at the allosteric site.
- Our results were in agreeance with a number of key residues involved in stabilising serotonin (R65, N101 & T154) at the orthogonal site. Novel residues (E102 & R219) could be exploited in drug design.
- At allosteric site, novel residues, R219, Q56, F222, Q53 and I139 were important in stabilising ginger actives.
- Ginger compounds scored highly in both sites.
    - Structural characteristics (flexibility, hydrophobicity, Hbond acceptors/donors) enable them to exploit complementary features in a binding pocket. Similar dual roles have been observed.

Conclusions / Future Directions

Analytical analysis

Quantification of ginger actives was conducted in a range of commercial ginger products to determine (Marx et al (2016)

Contents lists available at ScienceDirect

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Research paper

Determination of the concentration of major active anti-emetic constituents within commercial ginger food products and dietary supplements

Wolfgang Marx*, Elisabeth A. Isenring, Anna E. Lohning

Faculty of Health Sciences and Medicine, Bond University, Queensland, Australia

Future Work in Progress

Clinical:- A larger clinical trial has been accepted for funding (NHMRC, Feb 2017).

Mechanistic:- MD for pentameric ion channel in membrane.

Clinical Research Team / Collaborators / Funding Bodies

Research Team

Professor Liz Isenring
Head of Program, Nutrition & Dietetics Research Group
Bond University, Gold Coast, Australia

Dr. Wolfgang Marx
School of Allied Health,
LaTrobe University, Melbourne, Australia

Australian Government
National Health and Medical Research Council

Alexandra McCarthy, Princess Alexandra Hospital, QLD, Australia, Division of Cancer Services, Institute of Health and Biomedical Innovation, Brisbane, QLD, Australia; School of Nursing, University of Auckland, Auckland, NZ.

Karin Ried, Research Director, National Institute of Integrative Medicine

Dan McKavanagh, School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia; School of Nursing, University of Auckland, Auckland, NZ.

Luis Vitetta, Medlab Clinical Ltd, Sydney, NSW, Australia/University of Sydney, Sydney Medical School, Sydney, NSW, Australia.

Avni Sali, National Institute of Integrative Medicine, Melbourne, VIC, Australia

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Thank you!

Questions